HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE SEPARATION OF ISOMERS OF 9β-METHYLCARBACYCLIN (CIP-ROSTENE) AND THE ANALYSIS OF PHARMACEUTICAL SAMPLES

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SUMMARY

An high-performance liquid chromatography method was developed that separates (5Z)-9β-methyl-6α-carbaprostaglandin I_2 (9β-methylcarbacyclin; ciprostene) from several closely related isomers: (i) a double bond isomer at C-5, (ii) an epimer at C-15, and (iii) combinations of i and ii. The method uses a Zorbax ODS column with a mobile phase consisting of acetonitrile–buffer pH 2.5 at a ratio of 45:55 for determination of purity and 40:60 for optimum separation of isomers. Detection is measurement of absorbance at 200 nm. Peak heights are measured relative to butylparaben which is used as an internal standard. The method is linear, free from interferences from degradation of ciprostene and has a relative standard deviation of 0.5–1% for solutions at 60 μg/ml. This assay has been applied to bulk powders and solutions of the drug. Stressed chemical stability studies have shown that the C-15 allylic secondary alcohol group undergoes epimerization under acidic conditions in solution and oxidation in the solid and solution state under thermal stress or in the presence of oxidants.

INTRODUCTION

Ciprostene (9β-methylcarbacyclin; (5Z)-9β-methyl-6α-carbaprostaglandin I_2)[1] is a derivative of epoprostenol (prostaglandin I_2 or prostacyclin)[2] and is isolated as a calcium salt. Epoprostenol is a potent inhibitor of platelet aggregation, a vasodilator that is synthesized by the cell wall[3,4] and is chemically unstable and has a hydrolysis half-life of 15 min at pH 7.5 (ref. 5). The chemical stability of ciprostene is much better as it does not contain a vinyl ether group.

The structure of ciprostene is shown in Fig. 1 and can have several isomers because of the double bond at C-5, the secondary alcohols at C-11 and C-15 and the stereochemistry at C-8 and C-9. Epimerization at C-15 is commonly observed in prostaglandins under acidic conditions because of the allylic hydroxyl group. The intermediate carbonium ion is stabilized by delocalization of the charge. In the development of an analytical method for ciprostene, a specific procedure was desired
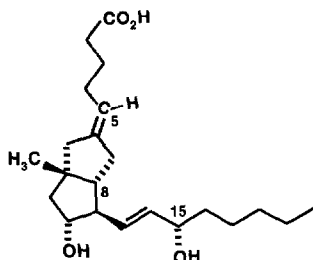
Fig. 1. Structure of ciprostene [(5Z)-9β-methyl-6α-carbaprostaglandin I₂, the configuration at C-15 is *R*).

that would separate the various isomers from each other. Separation of prostaglandin isomers has generally been best accomplished using high-performance liquid chromatography (HPLC), for examples see refs. 6–12. An HPLC method for ciprostene has not been previously reported. We discuss here an HPLC method for separating several closely related isomers of ciprostene.

EXPERIMENTAL

Instrumentation

The high-performance liquid chromatograph used for the separation was a microprocessor-controlled unit (Varian Model 5000, Walnut Creek, CA, U.S.A.) comprised of three solvent reservoirs. The selectable wavelength detector (Varian Model UV-5) was fitted with a 200-nm filter, an autosampler (Varian Series 8000) and a recorder (Houston Model Omniscribe). The flow-rate was 2.0 ml/min. The injection volume was 20 µl. The detector and recorder were set for 0.1 absorbance units full scale. Integration was completed on an in-house computer system.

The system used to obtain UV spectrums of chromatographic peaks was a component liquid chromatograph consisting of a pump (an Altex Model 110A, Beckman, Berkeley, CA, U.S.A.), a fixed loop injector (Rheodyne Model 7125, Berkeley, CA, U.S.A.), a high-speed spectrophotometric detector (Hewlett-Packard Model 1040A, Waldbronn, F.R.G.) containing a deuterium lamp and a photodiode array. The data were collected, processed and stored with a computer (Hewlett-Packard Model 85) and a flexible disc drive (Hewlett-Packard Model 82901M). The flow-rate was 2.0 ml/min. The injection volume was 200 µl.

Columns

Zorbax ODS columns (DuPont, Wilmington, DE, U.S.A.) (250 × 4.6 mm) were used. The packing is made by chemically bonding octadecylsilane groups to Zorbax silica and is spherical in shape with a 5–6 µm diameter.

Reagents and samples

The solvents were distilled-in-glass grade (Burdick and Jackson, Muskegon, MI, U.S.A.) and the buffers were ACS grade (Mallinckrodt, Paris, KY, U.S.A.). Samples of ciprostene (5Z,15S), the (5E,15S)-isomer, the (5E,15R)-isomer, the

(5Z,15R)-epimer and the 15-keto derivative were obtained from Paul Aristoff of the research laboratories of The Upjohn Company.

Sample preparation
Approximately 0.6 mg of ciprostene calcium was dissolved in 10 ml of internal standard solution for the purity assay. For the impurity assay, approximately 0.5 mg of drug was dissolved in 1 ml of mobile phase. Solution stress study samples were diluted with the appropriate amount of internal standard solution or mobile phase to approximately equal these concentrations. The sterile solutions of ciprostene at 1 mg/ml were diluted 1:9 with internal standard solution for the purity assay and used as is for the impurity assay. The internal standard solution was butyl paraben (Baker, Grade N.F.) made up in mobile phase at a concentration of 0.009 mg/ml.

RESULTS AND DISCUSSION

Initial development of a chromatographic procedure used ionization suppression with phosphoric acid and an acetonitrile:water mobile phase. Detection was at 200 nm because ciprostene has as chromophores only isolated double bonds and a carboxylic acid group. Phosphoric acid based buffers were preferred over acetic because of the absorption that acetic acid has below 230 nm. To evaluate the specificity
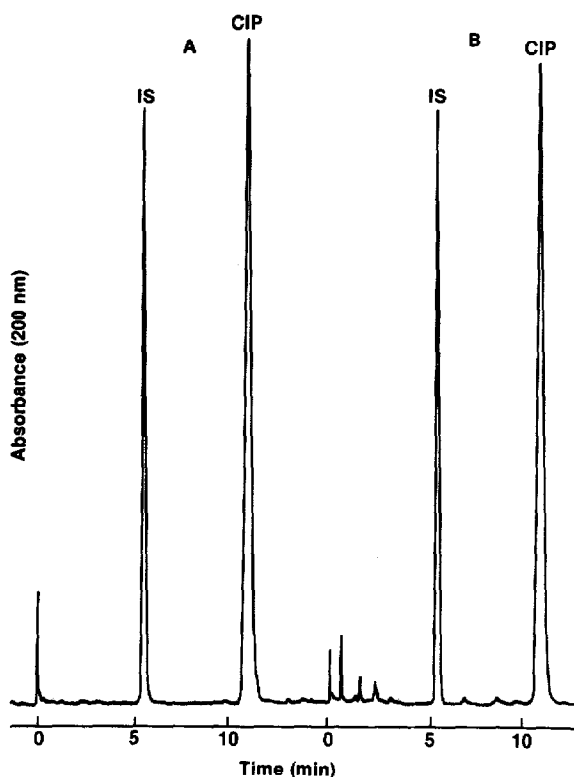

Fig. 2. Chromatogram showing a typical standard preparation (A) and a sample of sterile solution (B). IS = butylparaben (internal standard); CIP = ciprostene.

TABLE I

RELATIVE RETENTION TIMES OF THE ISOMERS

Mobile phase: acetonitrile–phosphate buffer pH 2.5 (40:60).

Isomer	Retention time (min)	Relative retention time
5E,15R	17	0.68
5Z,15R	18.5	0.74
8,9-diepi	19.5	0.78
5E,15S	21.8	0.87
Ciprostene (5Z,15S)	25	1.0

of the assay, a mixture containing (i) ciprostene (5Z,15S), (ii) the (5E,15S)-isomer, (iii) the (5E,15R)-isomer, (iv) (5Z,15R)-epimer, and (v) an unknown isomeric compound, tentatively identified as the 8,9-diepimer, was used. The unknown was concluded to be isomeric with ciprostene because of almost identical UV spectra and mass spectra. The ^{13}C NMR spectrum was consistent with the assignment as 8,9-diepimer but was not conclusive[13]. Two types of chromatographic separations were considered useful: (i) for evaluation of ciprostene purity, separation of the isomers from ciprostene was sufficient asnd (ii) for assessment of the impurities, the separation of all components was desired. For the purity assay, a mobile phase consisting of acetonitrile–phosphate buffer pH (45:55) was found to provide adequate resolution of the isomers from ciprostene in relatively short run times (Fig. 2). To resolve the isomers from each other, the acetonitrile concentration was decreased from 45 to 40% (Fig. 3). The relative retention times of the various isomers are shown in Table I. The most difficult separations are the separation of the (5E,15R)-ismer from the (5Z,15R)-isomer and the separation of the (5Z,15R)-isomer from the 8,9-diepimer. The effect of acetonitrile concentration in the mobile phase on the resolution of these two pairs is shown in Table II. The flow-rate was dropped as the acetonitrile concentration was increased to partially compensate for the reduced retention. The lower concentration of acetonitrile provides better resolution of the double bond isomer pairs *i.e.* 5E,15R–5Z,15R and 5E,15S–5Z,15S. The epimeric pairs at C-15 are well resolved under all the described conditions. The most difficult separation was that involving the (5Z,15R)-isomer and the 8,9-diepimer. The resolution between this pair is improved from 0.61 to 1.0 by increasing the acetonitrile concentration from 40 to

TABLE II

EFFECT OF MOBILE PHASE COMPOSITION ON RESOLUTION OF ISOMERS

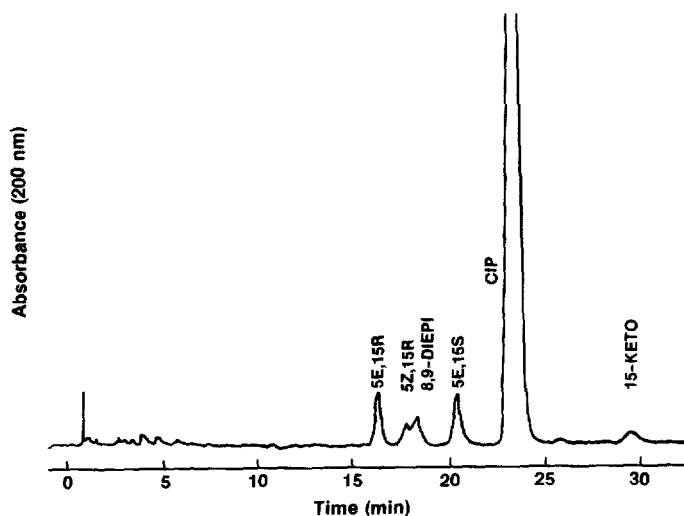| Acetonitrile (%)	Flow-rate (ml/min)	Retention time of ciprostene min	Theoretical plates for ciprostene	Resolution of (5Z,15R) and (5E,15R)	Resolution of (5Z,15R) and 8,9-diepimer
40	2.0	23.9	9065	2.05	0.61
45	1.5	17.3	8220	1.73	0.59
50	1.0	16.6	10,347	1.25	0.71
55	0.7	15.9	12,605	1.18	1.0

Fig. 3. Chromatogram showing resolution of the various isomers (see Fig. 1 for differences between isomers) for a mobile phase of acetonitrile–phosphate buffer pH 2.5 (40:60).

55% and dropping the flow-rate from 2 to 0.7 ml/min. However, substantial loss of resolution between the (5E,15S)-epimer and ciprostene (5Z,15S) occurs along with a decrease in resolution between the (5Z,15R)-isomer and the (5E,15R)-isomer. Hence, the optimum mobile phase depends on the extent of the various isomeric impurities. The (5E,15R)-isomer is an unlikely impurity in ciprostene because it has both the wrong double bond configuration and is the epimer at C-15. Hence, in many samples the resolution of this isomer may not be of concern. In general, the mobile phase with 40% acetonitrile was found to be best for separations where only small amounts of the epimeric isomers are present. Where large amounts of the C-15 epimer are present and the 8,9-diepimer is also present, the use of the mobile phase with 55% acetonitrile is more appropriate. The use of higher column temperatures or gradient elution did not have any advantages over the isocratic separation methods.

Some columns provided no resolution between the (15Z,15R) isomerm and the 8,9-diepimer under the conditions that had been determined to be optimum. An increase in mobile phase pH was known to improve resolution. An increase in apparent pH from 3.5 to 4.2 resulted in resolution of the pair of isomers. Higher pH values resulted in further improvement in resolution between these pairs of isomers but decreased the resolution between ciprostene and the (5E,15S)-isomer. At the optimum pH, the buffer capacity is quite low using phosphate as a buffer because the pH is too far from the pK_a values of phosphate (2.1, 7.2, 12.3). In the absence of sufficient buffering, the degree of ionization of analytes varies with concentration. As concentration varies within the chromatographic peak, the degree of sample ionization varies and peak broadening occurs[14]. The use of acetate (pK_a = 4.7) was found to give chromatographic peaks that were sharper. This allowed the separation to be accomplished in a shorter time. Another advantage of acetate is that it allows a larger amount of solubility-limiting calcium to be in solution. The problem with buffers for this pH region is that all are carboxylic acids and contribute to the background absorbance at 200 nm. The estimated reduction in detection limit is a factor

of 3 to 4 which can be compensated for by increasing the amount injected and the concentration of drug in the sample preparation. Some columns will require this alternate mobile phase of 42% acetonitrile and 0.5% acetic acid in water adjusted to pH 5 for resolution of the isomers.

Assay development and validation

Samples of pharmaceutical interest include bulk powder (ciprostene calcium) and a sterile solution. For the selection of an internal standard, a series of parabens were tested for chromatographic retention. Butylparaben with a relative retention time of 0.52 was well resolved from ciprostene and its isomers and was selected as an internal standard. Assays for bulk powders or sterile solution involved dissolving or diluting the sample in mobile phase containing butylparaben. Peak height ratios relative to the internal standard were linear from 10 to 140 μg/ml with a correlation coefficient > 0.999. The average recovery for drug spiked to a placebo containing all the ingredients in the formulation was 98.9% for concentrations ranging from 0.4 to 1.6 mg/ml. None of the formulation ingredients interferes with the assay. The relative standard deviation for the assay, in general, varies from 0.5 to 1.0%. A representative chromatogram is shown in Fig. 2.

Assay ruggedness

The stability of the drug in mobile phase is such that no significant change in drug concentration was observed within a two day period. At least three different columns have been used and the reproducibility between columns was such that the isomers were separated from each other with all the columns. The resolution between the (5Z,15R)-isomer and the 8,9-diepimer is such that only columns with a high plate count can achieve an adequate separation, hence, some difficulty with old columns may be expected.

Degradation

To determine if the assay separated potential degradation products from ciprostene the drug was subjected to conditions designed to force degradation. In the solid state, the drug was: (i) heated at 50°C and 70°C, (ii) placed in a 75% relative humidity chamber at 25°C, and (iii) exposed to fluorescent light (30 W at a distance of 30 cm). The duration of these studies was four weeks and samples that had degraded at least 10% were used to assess the specificity of the method. A peak at a relative retention time of 1.24 *vs.* ciprostene was the major peak forming. This peak was tentatively identified as the 15-keto derivative formed as a result of oxidation of the C-15 alcohol to a ketone. The basis for this conclusion is the reduced polarity of the compound relative to ciprostene, the absorbance band at 233 nm consistent with a α,β-unsaturated ketone, and identical retention to that of an authentic sample. Other small, unidentified peaks were observed, usually with short retention times, but none were observed to interfere with the ciprostene peak. The drug most rapidly degraded under fluorescent light. Based on the appearance of the 15-keto compound, oxidation of the compound is a major route of degradation in the solid state and the fluorescent light would be expected to rapidly accelerate degradation.

In the solution state, solutions of the drug were prepared: (i) in water at 25°C, (ii) in water and exposed to fluorescent light, (iii) in 0.1% hydrogen peroxide, (iv) in

solution with perchlorate salts of Fe^{3+}, Cu^{2+} and Zn^{2+} at a ten to one molar ratio of metal to drug, and (v) at pH 8 at 70°C and pH 1 at 25°C. Again, the duration of the study was four weeks. Degradation occurred most rapidly at low pH as a result of epimerization at the C-15 alcohol and under oxidative conditions (*i.e.* hydrogen peroxide) which formed the 15-keto derivative. Epimerization also occurred in the $Fe(ClO_4)_3$ solution. The Fe^{3+}, acting as a Lewis acid in this situation, promoted epimerization. The identification of the degradation products is at an early stage at this time. The assay is capable of separating potential degradation products from 9β-methyl-carbacyclin and can be used to assess the chemical stability of bulk and solution samples.

ACKNOWLEDGEMENTS

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